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Hyperthermic Enhancement of Cytotoxicity and Increased Uptake of *cis*-Diamminedichloroplatinum(II) in Cultured Human Esophageal Cancer Cells

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Thermal enhancement of cytotoxicity of *cis*-diamminedichloroplatinum(II) (CDDP) has been well recognized and applied clinically to chemotherapy of various malignancies, but its fundamental mechanism remains to be elucidated. In order to obtain a clue to this mechanism, we analyzed the effect of hyperthermia on the uptake and subsequent distribution of [^{195m}Pt]CDDP in two lines of esophageal cancer cells (KYSE-150 and KYSE-170) established from clinical patients. First, we observed a significant increase in [^{195m}Pt]CDDP uptake by both types of cells at increasingly higher temperatures. The incorporated CDDP was distributed between the nucleus and the cytosol at a ratio of approximately 3:1, and the ratio remained the same at various temperatures. The CDDP was found in all four molecular fractions, i.e., DNA, RNA, protein, and TCA-soluble, with a slight preference for DNA at higher temperatures. Enhancement of cytotoxicity required simultaneous, and not sequential, treatments with CDDP and hyperthermia; hyperthermia after CDDP treatment increased the efflux of CDDP from the cells, and rather reduced the cytotoxicity of CDDP. These results suggest that thermal enhancement of the cytotoxicity of CDDP is caused mainly by acceleration of the drug entry into the cell, probably due to increased permeability, and a consequent increase in the amount of CDDP binding to DNA. This mechanism gives support for clinical trial of simultaneous treatment with CDDP and hyperthermia.

Key words: Esophageal cancer — *cis*-Diamminedichloroplatinum(II) — Hyperthermia — [^{195m}Pt]CDDP

cis-Diamminedichloroplatinum(II) (CDDP[6]) is one of the few chemotherapeutics that are clinically effective against human esophageal cancers. In fact, CDDP proved to be effective in about a quarter of patients suffering from esophageal cancer.[1] The cytotoxic effect of CDDP has been shown to be enhanced by combination with hyperthermia clinically[2,3] and experimentally.[4–12] The esophagus is an organ which can be treated by local heating using a special intraluminal hyperthermia applicator. From clinical experience, simultaneous treatment with heat and CDDP is known to be more effective than sequential treatment, but the basis for this empirical finding has not been clearly understood as yet. For better use of this thermal enhancement effect, an understanding of its fundamental mechanism is required.

Four possible modes of interaction between anticancer drug and hyperthermia have been proposed[10]: increase in drug uptake by the cell, alteration of the intracellular distribution of the drug, alteration of its metabolism, and enhancement of the drug action on DNA. In order to obtain a better insight into the thermal enhancement of CDDP cytotoxicity, we examined changes in the level and intracellular distribution of CDDP induced by heat treatment in cultured human esophageal cancer cells. We prepared and used ^{195m}Pt-labeled CDDP, which made possible very sensitive quantitation of the drug.

MATERIALS AND METHODS

Drugs [^{195m}Pt]CDDP was synthesized from ^{195m}Pt generated by Kyoto University Research Reactor. Specific radioactivity of the preparation was $>7.4\times10^6$ Bq/mg ($T_{1/2}$=4.02 days), and the chemical purity was 99.7%, as reported previously.[13] Unlabeled CDDP was a gift from Nippon Kayaku Co. Ltd.

Cell culture Human squamous cancer cell lines, KYSE-150 and KYSE-170, were established from surgical specimens of esophageal cancers in our Department.[14] Both lines of cells were maintained in a mixed medium of alpha-MEM and RPMI1640, supplemented with 5% fetal bovine serum (HyClone), penicillin G (100 units/ml), and gentamycin (5 μg/ml). The doubling time was 19–23 h at 37°C in a 5% CO_2/95% air atmosphere, and the plating efficiency was 10–40% for both types of cells.

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[6] The abbreviations used are: CDDP, *cis*-diamminedichloroplatinum(II); TCA, trichloroacetic acid; MEM, minimal essential medium; HEPES, *n*-(2-hydroxyethyl)piperazine-*n*′-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

Assay of cell survival after treatments with CDDP and hyperthermia Twenty-four hours before thermal and CDDP treatment, exponentially growing KYSE-150 or KYSE-170 cells were placed in 25-cm^2 flasks containing complete medium. For simultaneous thermal and CDDP treatments, the medium was replaced by serum-free medium containing CDDP at a concentration of 2.5–20 μM, and the flask was submersed horizontally in a water bath kept at 37°C, 40°C or 42°C (±0.1°C). For control of the sequence of treatments, another flask of exponentially growing KYSE-150 cells was incubated first with CDDP in serum-free medium for 1 h, and then, after rinsing of the cells three times with MEM containing 5% calf serum and addition of fresh medium, submersed in a water bath at 42°C for 1 h. After exposures to CDDP and heat, the cells were trypsinized, and appropriate portions were replated into 60-mm dishes. The dishes were incubated at 37°C for 10–14 days until colonies became macroscopically visible. The colonies were stained with crystal violet and counted. Triplicate dishes were used for each experimental point.

Assay of CDDP uptake by the cell Exponentially growing cells were treated with CDDP and/or hyperthermia as described above, except that nonradioactive CDDP was replaced by [^{195m}Pt]CDDP (8.3 μM). The cells were washed three times with MEM containing 5% calf serum, then trypsinized and collected by centrifugation at 1500 rpm for 4 min. The amount of [^{195m}Pt]CDDP incorporated into the cells was determined by measuring the radioactivity in the cell suspension.

Analysis of intracellular distribution of platinum After treatment with [^{195m}Pt]CDDP and hyperthermia, the cells were swollen in homogenization buffer [5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES-NaOH (pH 7.1), 0.5 mM PMSF, 5 μg/ml leupeptin], and subjected to twenty strokes with a micro tissue grinder (Weaton). Disruption of >90% cells was confirmed microscopically. The homogenate was centrifuged at 2500 rpm for 5 min, and the radioactivity in the pellet (the "nucleus") or the supernatant (the "cytosol," containing membranes and extranuclear particles) was measured by a gamma counter (Aloka NDW 351).

Analysis of platinum incorporated into molecular fractions The cells treated with [^{195m}Pt]CDDP and hyperthermia, trypsinized and collected as above, were subjected to isolation of four fractions (DNA, RNA, protein, and TCA-soluble) by Schneider's method.[15] [^{195m}Pt]-CDDP incorporated into each fraction was quantified by measuring the radioactivity.

Assay of DNA DNA was assayed by the method of Thomas and Farquhar[16] using 3,5-diaminobenzoic acid.

RESULTS

The cytotoxic action of CDDP was substantially enhanced by elevation of temperature from 37°C to 40°C and further to 42°C at all concentrations of CDDP tested in both KYSE-150 and KYSE-170 cells (Fig. 1).

We then analyzed the fate of [^{195m}Pt]CDDP incorporated into the cells in two types of subcellular fractions, i.e., compartmental (nucleus and cytosol) and molecular fractions (DNA, RNA, protein, and TCA-soluble). Incorporation of CDDP into the nucleus and the cytosol increased almost in parallel as the temperature was raised from 37°C to 40°C or 42°C, keeping the nucleus vs. cytosol ratio and the nuclear proportion almost constant (Table I); about 73% and 78% of the incorporated platinum was accumulated in the nuclei of KYSE-150 and KYSE-170 cells, respectively, at all three temperatures tested.

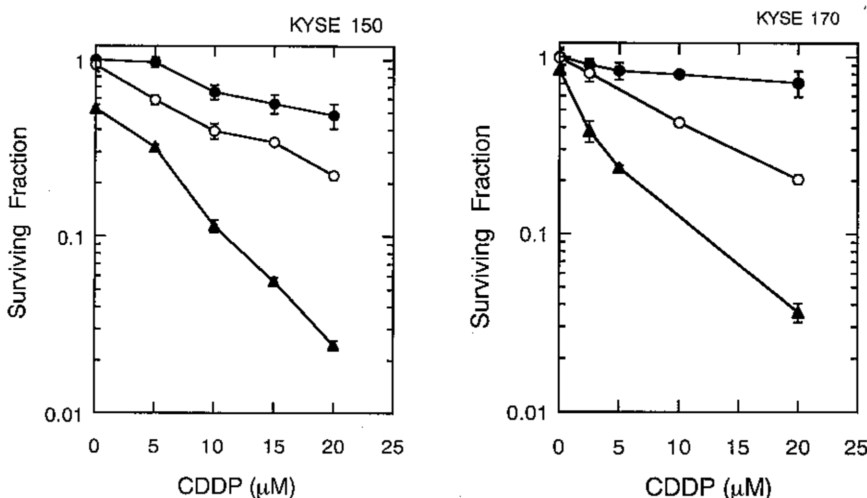

Fig. 1. Temperature-dependence of CDDP cytotoxicity to human esophageal cancer cells. Exponentially growing KYSE-150 or KYSE-170 cells were exposed to various concentrations of CDDP for 1 h at 37°C (●), 40°C (○), or 42°C (▲). Each point represents the mean of three independent determinations ± SE (bars).

Table I. Subcellular Distribution of CDDP in KYSE-150 and KYSE-170 Cells

Cells	Temperature	Radioactivity (cpm/10^6 cells) recovered in Nucleus (a)	Cytosol (b)	Total (c)	a/b	a/c
KYSE150	37°C	157±2	59±9	216±3	2.66	0.73
	40°C	247±13*	92±9	339±20	2.68	0.73
	42°C	385±31**	138±9*	523±40**	2.80	0.74
KYSE170	37°C	391±94	118±26	509±120	3.31	0.77
	40°C	654±95*	174±8*	828±10*	3.76	0.79
	42°C	899±73**	250±21**	1149±93**	3.60	0.78

The cells were treated with 8.3 μM [^{195m}Pt]CDDP for 1 h at 37°C, 40°C, or 42°C. Nuclei were isolated, and the radioactivity in the nucleus and the cytosol was determined with a gamma counter. Mean±SD.
* $P<0.05$, ** $P<0.10$ (Student's t test); the level of significance of the difference as compared with 37°C.

Table II. CDDP Uptake into DNA, RNA, Protein, and TCA-soluble Fractions

Cells	Temperature	Radioactivity (%) recovered in DNA	RNA	Protein	TCA-soluble	Total
KYSE-150	37°C	6.5±0.8	6.6±1.8	52.3±3.1	34.7±3.6	100.0
	40°C	15.1±2.6	11.1±3.0	90.6±15.8	50.7±1.8	167.5±16.7
	42°C	27.9±8.0	14.8±4.0	108.5±16.9	66.6±4.8	217.7±20.1
KYSE-170	37°C	5.0±1.2	10.9±1.6	48.7±2.8	35.4±3.2	100.0
	40°C	7.5±0.8	13.1±0.5	65.9±1.1	46.1±2.7	132.7±1.8
	42°C	12.8±5.3	16.2±0.9	92.8±15.1	77.6±3.8	199.4±25.2

The cells were treated with 8.3 μM [^{195m}Pt]CDDP for 1 h at 37°C, 40°C, or 42°C. Then, four molecular fractions were isolated and examined for radioactivity as described under "Materials and Methods." Total radioactivity incorporated into the four fractions of each cell line at 37°C was taken as 100%. Mean±SD.

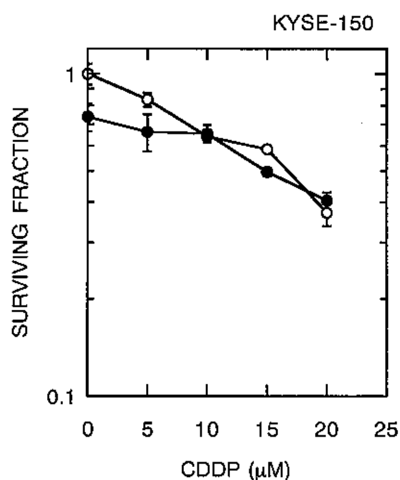

Fig. 2. Effect of hyperthermia on the cytotoxicity of preceding CDDP treatment in KYSE-150 cells. After treatment at various concentrations of CDDP for 1 h, cells were treated with (●) or without (○) heat (42°C, 1 h).

Incorporation of [^{195m}Pt]CDDP into the four molecular fractions prepared by Schneider's method also increased with elevation of the temperature from 37°C to 40°C and to 42°C in both KYSE-150 and KYSE-170 cells (Table II). Among the four fractions, the incorporation into the DNA fraction increased most markedly, i.e., 4.3- and 2.6-fold in KYSE-150 and KYSE-170 cells, respectively, at 42°C compared with 37°C.

When thermal treatment was performed immediately after CDDP treatment, a weaker-than-additive effect was observed in KYSE-150 cells (Fig. 2). In both lines of cells treated with CDDP followed by hyperthermia, the intracellular level of [^{195m}Pt]CDDP was lower than the level of simple CDDP treatment (Table III), suggesting that the efflux of CDDP was induced by the thermal treatment.

DISCUSSION

In the present study, we approached the mechanism of thermal enhancement of CDDP cytotoxicity by analyz-

Table III. Effects of Various Combinations of CDDP and Heat Treatments on CDDP Uptake by KYSE-150 and KYSE-170 Cells

Treatment	[^{195m}Pt]CDDP (cpm/10^6 cells) incorporated into	
	KYSE-150	KYSE-170
CDDP	247.9±6.2	515.9±17.8
CDDP→HT[a]	211.8±14.9	428.5±11.5
HT→CDDP[b]	259.2±14.9	676.9±50.5
CDDP+HT[c]	536.3±29.4	990.7±61.6

a) CDDP (8.3 *μM*, 37°C, 1 h) treatment, followed immediately by heat treatment (42°C, 1 h).
b) CDDP treatment, immediately after heat treatment.
c) Simultaneous treatment with CDDP (8.3 *μM*, 37°C, 1 h) and heat.
Mean±SD.

ing incorporation and subsequent distribution of [^{195m}Pt]-CDDP in two human esophageal cancer cell lines. The use of [^{195m}Pt]CDDP made possible quantitation of as little as 0.05 ng of platinum, that is, an amount several-fold less than that detectable by the conventional atomic absorption method. This method, however, did not allow us to distinguish the types of adducts, i.e., monofunctional or bifunctional.

An increase in cellular uptake with hyperthermia has so far been aocumented for various anticancer drugs.[17-20] As for CDDP, however, the effect of hyperthermia on the drug uptake remains unclear; Mansouri *et al.*[21] reported that elevation of the temperature from 37°C to 43°C increased the incorporation of platinum into RIF-1 cells, whereas Herman *et al.*[22] observed no change in the amount of platinum entering SCC-25 cells at elevated temperatures. In these reports, platinum was measured by atomic absorption spectrophotometry. Our present results using radiolabeled CDDP support the former view of an increased uptake at higher temperatures.

The increase in [^{195m}Pt]CDDP uptake with elevation of temperature occurred in parallel in the nucleus and the cytoplasm (Table I) and in all four molecular fractions isolated by Schneider's method (Table II). These results indicate that the increased uptake of CDDP was due to a general increase in the permeability of cell membranes and passive diffusion. It is noteworthy that, while the total level of intracellular CDDP increased 2.4 and 2.3-fold at 42°C compared with 37°C (Table I), the level of CDDP bound to DNA increased more markedly, 4.3- and 2.6-fold, at 42°C compared with 37°C in KYSE150 and KYSE170 cells, respectively (Table II). This preference of CDDP for DNA binding may be ascribable to partial losening of double strands of DNA at higher temperatures. On the other hand, when hyperthermia was performed before CDDP, a slight increase in cellular CDDP uptake was observed (Table III), indicating persistence of a membrane change caused by hyperthermia during the CDDP treatment.

DNA is widely believed to be the critical target for CDDP, and its cross-linking is considered as the lethal lesion. Our results that hyperthermia enhanced the entry of CDDP into the cell, and thereby increased the binding to DNA suggest that an increase in the number of DNA crosslinks plays a central role in the thermal enhancement of CDDP cytotoxicity.

There are several reports suggesting the importance of the sequence of treatments for the thermal enhancement of CDDP effect.[23,24] Maximal cytotoxicity of CDDP to HA1 Chinese hamster ovary cells was observed when hyperthermia was performed simultaneously with drug treatment *in vitro*.[5] For BT4A neurogenic rat tumor cells, maximal enhancement *in vivo* was obtained by an i.p. injection of CDDP under simultaneous local heating.[25] Similarly, Overgaard *et al.*[26] reported that simultaneous applications of CDDP and hyperthermia caused an apparently synergistic effect, whereas their sequential administration with a long interval between the two treatments resulted in only an additive effect. Our finding of marked enhancement of cytotoxicity by simultaneous treatment, but not by sequential treatments (Fig. 2), is in accord with these observations. It should be noted that hyperthermia after CDDP apparently resulted in efflux of the drug, thereby lowering the drug concentration in the cell (Table III), and consequently the cytotoxicity.

All these results indicate that simultaneous treatment with CDDP and hyperthermia is essential for effective killing of tumor cells, and support the clinical view that CDDP should be administered during hyperthermic therapy. This does not exclude the possibility that hyperthermia may also modify various factors, such as local blood flow, pH, and tissue O_2 concentrations, and modulate the *in vivo* effect of CDDP in a complex manner. Further analyses of the mechanism of thermal enhancement of CDDP entry into the cell, the relationship between the level of DNA-bound platinum and cytotoxicity, and cellular transport and metabolism of CDDP in various cells with different sensitivities will help to establish the molecular basis of the effect, and perhaps lead to applications of the combination therapy to other malignancies.

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